Leukocyte - Cerebral Endothelial Cell Interactions in the Brain Lead to Anxiety-like Behaviour in Experimental Colitis

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Abstract

BACKGROUND

Behavioral comorbidities, such as anxiety and depression, are a prominent feature of IBD. The signals from the inflamed gut that cause changes in the brain leading to these behavioral comorbidities remain to be fully elucidated. We tested the hypothesis that enhanced leukocyte - cerebral endothelial cell interactions in experimental colitis initiate neuroimmune activation leading to anxiety-like behaviour.

METHODS

Male and female mice treated with dextran sodium sulfate were studied at the peak of acute colitis. Circulating leukocyte populations were determined using flow cytometry. Leukocyte - cerebral endothelial cell interactions were examined using intravital microscopy in mice treated with anti-integrin antibodies. Brain cytokine and chemokines were assessed using a multiplex assay in animals treated with anti- $\alpha 4\beta 7$ integrin. Anxiety-like behavior was assessed using an elevated plus maze in animals after treatment with an intracerebroventricular injection of interleukin 1 receptor antagonist.

RESULTS

The proportion of classical monocytes expressing $\alpha 4\beta 7$ integrin was increased in peripheral blood of mice with colitis. An increase in the number of rolling and adherent leukocytes on cerebral endothelial cells was observed, the majority of which were neutrophils. Treatment with anti- $\alpha 4\beta 7$ integrin significantly reduced the number of rolling leukocytes. After anti-Ly6C treatment to deplete monocytes, the number of rolling and adhering neutrophils were significantly reduced in mice with colitis. Interleukin-1 β levels were elevated in the brain and treatment with anti- $\alpha 4\beta 7$ significantly reduced them. Enhanced anxiety-like behaviour in mice with colitis was reversed by treatment with interleukin 1 receptor antagonist.

CONCLUSIONS

$\alpha 4\beta 7$ integrin expressing monocytes direct the recruitment of neutrophils to the brain vasculature, leading to elevated cytokine levels that mediate anxiety-like behaviour in experimental colitis.

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the gastrointestinal (GI) tract with a relapsing and remitting time course and a multifactorial (i.e. genetic, environmental and immune) etiology [1, 2]. Patients experience abdominal pain, diarrhea, rectal bleeding and weight loss during disease exacerbations, and may continue to experience pain and altered gut function, even when inflammation has resolved [3-5]. Behavioral comorbidities are a prominent feature of IBD. Individuals with IBD experience depression, anxiety, fatigue, decreased sociability, sleep disturbances and cognitive dysfunction [6-10]. These cognitive, emotional and behavioural abnormalities occur more commonly in women. They are observed during active disease and when it is in remission and have a significant

negative impact on quality of life. Despite significant advances in understanding the pathogenesis of IBD, the signals from the inflamed gut that bring about changes in the brain that lead to behavioral comorbidities remain to be fully elucidated.

The central nervous system (CNS) senses and integrates signals originating from the GI tract, providing a dynamic imprint of the state of the gut [11, 12]. Signaling from the gut to the brain is complex, and involves neural, humoral, microbial and cellular mediators [13, 14]. In addition to peripheral neural pathways and circulating factors that directly and indirectly access the CNS, it is becoming increasingly clear that the immune system communicates with the brain [14-16]. When inflammation occurs in the body, activated immune cells produce mediators, including cytokines, that communicate changes in peripheral immunity to the CNS via all these pathways. The cellular pathways include the direct trafficking of activated immune cells to the brain, which then sets in motion neuroimmune and cellular mechanisms that ultimately alter excitability of the CNS, leading to behavioral changes [14, 16-18]. We and others have documented that animal models of IBD recapitulate many of the behavioural changes observed in patients, including anxiety-like and depressive-like behaviors [19-25]. Altered neural and immune signaling in the CNS have also been demonstrated in these animal models of IBD [17, 20, 26-28].

The molecular signaling mechanisms that guide trafficking of activated immune cells to the brain in IBD and experimental colitis are not well understood. In contrast, trafficking of immune cells to the inflamed gut in IBD has received significant experimental and clinical attention as a therapeutic target to decrease gut inflammation [29-31]. Specifically, the integrin, $\alpha 4\beta 7$ was found to be a key regulator for intestinal homing of lymphocytes through binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is upregulated in response to intestinal inflammation [32, 33]. This important observation led to the development and clinical use of the $\alpha 4\beta 7$ monoclonal antibody vedolizumab for treating IBD [34, 35]. However, it has become clear that $\alpha 4\beta 7$ is expressed not only on gut-homing lymphocytes, but also on monocyte subsets that can regulate intestinal inflammation [36, 37]. The potential role of $\alpha 4\beta 7$ integrin-expressing leukocytes in immune cell trafficking to the brain remains to be shown, but it is noteworthy that IBD patients treated with vedolizumab not only have improved colitis, but also an improvement in sleep quality and mood [38], suggesting that $\alpha 4\beta 7$ integrin inhibition can regulate behavior, possibly by altering immune cell trafficking to the brain.

We investigated immune cell trafficking in the well-characterized animal model of IBD, dextran sodium sulphate (DSS)-induced colitis [39]. We tested the hypothesis that enhanced leukocyte - cerebral endothelial cell interactions occur in the brain in experimental colitis, mediated by $\alpha 4\beta 7$ integrin, to initiate neuroimmune activation and lead to anxiety-like behaviour. We show that $\alpha 4\beta 7$ integrin-expressing monocytes within the circulation direct the recruitment of neutrophils to the brain vasculature, leading to elevated levels of the proinflammatory cytokine interleukin (IL)-1 β that mediates anxiety-like behaviour in this model of experimental colitis.

Materials And Methods

Animals

Female (16-22 g) and male (22-30 g) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) aged 7-8 weeks on arrival, were group housed (3-4 mice per cage), under a 12 h light–dark cycle (lights off 19:00) in plastic sawdust floor cages (22°C, 40% humidity, standard laboratory chow and water ad libitum) in a specific pathogen-free facility. After one week of acclimatization, cages of mice were randomly assigned to treatment groups. All experimental procedures were approved by the Health Sciences Animal Care Committee of the University of Calgary and were carried out in accordance with the guidelines of the Canadian Council on Animal Care (Protocol #'s AC17-0093, AC15-0129).

DSS-induced colitis

Mice were given DSS (40–50 kDa, Affymetrix, Cleveland, OH, USA) ad libitum in their drinking water (2.5%-3.5% wt/vol); on day 5 this was replaced with tap water until day 7. Control mice received tap water alone for 7 days. Body weight was measured three times per week. Day 7 has previously been determined to be the peak of colonic inflammation in this model [19].

In all cases, mice were euthanized by cervical dislocation under isoflurane or ketamine-xylazine anesthesia on day 7. Body weight score was calculated as the % weight loss on day 7 from the initial body weight on day 0 (0 = 0 %, 1 = <0–≤5 %, 2 = >5–≤10 %, 3 = >10–≤15 %, 4 = >15 %). The colon was dissected and examined by a blinded observer for macroscopic evidence of colitis. Colon length score was calculated as a % of control colon length, with the average control length in females being 6.0 cm and in males 7.4 cm: (0 = 85-100%, 1 = 75-84 %, 2 = 65-74 % and 3 < 65 %). The presence (score = 1) or absence (score = 0) of adhesion, erythema, gross fecal blood and diarrhea was recorded. A total damage score was calculated for each animal comprising, body weight score, adhesion, colon length score, erythema score, fecal blood score, diarrhea score, length of inflamed colon as % of total length, ulcer length and bowel thickness (mm). The macroscopic damage score is presented as mean ± SEM for ease of comparison with the literature, while acknowledging that these are non-parametric values.

Flow cytometry

To determine the proportions of leukocyte population in colitis, we performed a flow cytometric analysis. After cervical dislocation, blood was immediately withdrawn by cardiac puncture. A whole blood staining method was used to investigate the phenotypic profile of peripheral blood leucocytes. In order to block non-specific binding to Fc III/II receptors, 100 µL of anticoagulated whole blood were added to a 5 mL polystyrene tube and incubated with anti-CD16/CD32. Following incubation at room temperature for 15 min, a predetermined optimum concentration of desired fluorochrome-conjugated primary antibodies was added and incubated for 30 min. Red blood cells were lysed by adding 2 mL of Ammonium-Chloride-Potassium lysis buffer. Following incubation at room temperature for a further 10 min, cells were washed twice in staining buffer by centrifugation at 500xg for 10 min. Samples were acquired either using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) or Attune™ Acoustic Focusing flow cytometer (Applied Biosystems, Mainway, Burlington, ON, Canada). Data were analyzed using FlowJo®

software (Treestar, Ashland, OR, USA). Flow cytometry dot-plots showing the gating strategy used in the identification of $\alpha 4\beta 7$ expressing monocytes and neutrophils in mouse blood are shown in Supplementary Figure 1. The following antibodies were obtained from sources indicated: anti-mouse CD16/CD32 (93), anti-mouse Ly6C (HK1.4), (ThermoFisher Scientific, Waltham, MA, USA). Anti-mouse CD11b (M1/70), anti-mouse Ly6G (1A8), anti-mouse CD3 ϵ (145-2C11), anti-mouse Integrin $\alpha 4\beta 7$ (DATK32) (BioLegend, San Diego, CA, USA). Data are shown as mean $\pm$ standard error of the mean (SEM) of 5 mice per group. For comparisons between two groups, an unpaired Student's t-test was performed (GraphPad Prism version 9, GraphPad, San Diego, CA, USA). A P value of ≤ 0.05 was considered significant.

Intravital microscopy

To examine leukocyte – endothelial interactions in colitis, we performed intravital microscopy using previously published approaches [20]. On day 7 of colitis, mice were anesthetized using a ketamine and xylazine mixture (i.p.; 200mg/kg and 10mg/kg, respectively). The tail vein was cannulated for administration of dyes and conjugated antibodies for imaging. The skin was bluntly dissected from the skull and the parietal bone thinned to approximately 30 μ m using a high-speed dental drill, resulting in an intact cranial window over the parietal cortex of approximately 5 mm x 5 mm [40]. The window was covered with a drop of saline and the mouse placed on the microscope stage.

Dyes or conjugated antibodies were administered i.v. immediately before imaging: Rhodamine-6G (0.225 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was used to visualize all leukocytes [17], phycoerythrin (PE)- or allophycocyanin (APC) -labelled CD-31 (390; 2 μ g/mouse; eBioscience; catalog #17-0311-82 (APC)- #12-0311-81, (PE)) was used to label cerebral endothelial cells, PE-labelled Ly6G (1A8, 2 μ g/mouse; eBioscience; catalog #12-9668-80) was used to label neutrophils [17], APC-labelled Ly6C (HK1.4; 2 μ g/mouse; eBioscience; catalog #17-5932-80) was used to label monocytes, and APC-labelled MAdCAM-1 (MECA-367; 2 μ g/mouse; Biolegend, San Diego, CA, USA; catalog #120711) was used to label MAdCAM-1.

For the depletion of specific cells, mice with colitis were i.p. administered antibodies or isotype control, 18-22h prior to the imaging experiment. Circulating neutrophils were depleted using anti-Ly6G (1A8; 200 μ g/mouse; Bio X Cell, Lebanon, NH, USA; catalog #BE0075-1), while the controls received rat IgG2a antibody (200 μ g/mouse; Bio X Cell; catalog #BE0089). The efficiency of the mAb anti-Ly6G to specifically deplete classical monocytes in C57BL/6 mice was confirmed using flow cytometric analysis as shown in Supplementary Figure 2. Anti- $\alpha 4$ integrin (PS/2; 200 μ g/mouse; Bio X Cell; catalog #BE0071) and anti- $\alpha 4\beta 7$ integrin (DATK32; 200 μ g/mouse; Bio X Cell; catalog #BE0034) were administered to investigate the role of integrins in leukocyte recruitment. The controls were administered rat IgG2b (200 μ g; Bio X Cell; catalog #BE0090) and rat IgG2a, respectively. Other mice were administered anti-MAdCAM-1 (MECA-367; 200 μ g/mouse; Bio X Cell; catalog #BE0035) or rat IgG2a. Circulating monocytes were depleted using anti-Ly6c (HK1.4; 100 μ g/mouse; eBioscience; catalog #16-5932-85), with controls receiving rat IgG2c (100 μ g; Biolegend; catalog #400710). The efficiency of the mAb anti-Ly6C to

specifically deplete classical monocytes in C57BL/6 mice was confirmed using flow cytometric analysis as shown in Supplementary Figure 3. We have previously used this anti- $\alpha 4$ integrin neutralizing antibody strategy to prevent monocyte adhesion to cerebral endothelial cells [41].

Leukocyte-endothelial interactions were imaged in the parietal cortex using a Quorum WaveFX spinning disk confocal microscope (Quorum Technologies, Puslinch, ON, Canada) driven by Volocity 6.1 acquisition software (PerkinElmer, Waltham, MA, USA). Labeled cells were imaged using 561, or 635 nm laser excitation and visualized with the appropriate long pass filters using a 20X/0.95 NA water objective. A 512 X 512 pixel back-thinned EMCCD camera (Model C9100-13; Hamamatsu Corp., Hamamatsu City, Japan) was used for fluorescence detection. 3-5 venules (17-40 μm diameter) were recorded for 1 min and data averaged per mouse. Rolling leukocytes were classed as those that moved at a velocity less than that of an erythrocyte down a 100 μm segment of vessel. Adherent cells were classed as those that were stationary for 30 sec or longer within the 100 μm segment of vessel [17]. Data are shown as mean $\pm$ standard error of the mean (SEM). For comparisons between two groups, an unpaired Student's t-test was performed (GraphPad Prism). A P value of ≤ 0.05 was considered significant. A total of 90 animals were successfully used in 32 cohorts. Four outliers were identified using the Grubbs' test and were removed.

Cytokine measurements

To delineate the importance of leukocyte - cerebral endothelial cell interactions in initiating neuroimmune activation in the brain, we measured prefrontal cortical cytokine levels 7 days after DSS treatment. On day 4 and 6 of DSS treatment, the control group (n=6) was administered sterile PBS 10mL/kg, i.p. while the DSS-treated mice were given either control IgG2a antibody (200 μg /mouse, IP; Bio X Cell; catalog #BE0089, n=6), or anti- $\alpha 4\beta 7$ integrin antibody (200 μg /mouse, IP; Bio X Cell; catalog #BE0034, n = 6) to investigate the role of integrins in cytokine changes in the brain. On day 5, DSS administration was stopped, and all mice were given tap water. In the morning of day 7, mice were anesthetized with isoflurane and transcardially perfused with cold PBS buffer for 5 min while under anesthesia. The prefrontal cortex was microdissected and immediately snap-frozen in liquid nitrogen and stored at -80°C until processing. The isolated cortical tissue was homogenized using a Micro-Tube homogenizer in tissue protein extraction buffer (RIPA buffer containing protease inhibitor cocktail). The homogenate was centrifuged at $15000 \times g$ for 15 min at 4°C , and supernatants were collected and stored at -20°C until analysis. Mouse cytokines (IFN γ , IL-1 β , GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-12(p70), CCL2, TNF α) were simultaneously measured in tissue homogenate samples using a mouse MILLIPLEX kit (Millipore, Burlington, MA, USA) according to the manufacturer's protocol. The multiplexing analysis was performed using the Luminex 100 system (Luminex®, Austin, TX, USA) by Eve Technologies Corporation (Calgary, AB, Canada). Total protein concentration in tissue homogenates was quantified using a BCA Protein Assay kit (ThermoFisher) according to the manufacturer's instructions. Results were expressed as pg of analyte/mg of protein. All data are shown as mean $\pm$ standard error of the mean (SEM) of 6 mice per group. For comparisons between groups, an analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test was performed (GraphPad Prism). A P value of ≤ 0.05 was considered significant. The Grubbs' test was used to identify and exclude potential statistical outlier data points.

Intracerebroventricular (ICV) cannulation and infusion.

To assess whether blocking elevated IL-1 β levels in the brain would alter behavior we administered IL-1ra intracerebroventricularly. Mice were anesthetized with isoflurane and a guide cannula (23G, 8 mm) was implanted under stereotaxic guidance above the right lateral ventricle (from Bregma: +0.5 mm, lateral: +1.0 mm, depth: +1.4 mm). Mice were given analgesic treatment (buprenorphine, provided by the Health Science Animal Resource Centre, Calgary, AB, Canada, 0.05 mg/kg subcutaneously before and after the surgery) and allowed to recover for 5 days, after which DSS was administered in drinking water for another 5 days as described above. Body weight was recorded pre-and post-surgery, as well as during and after DSS administration/ICV infusions.

At the onset of DSS administration, both control and DSS-treated mice were infused ICV (0.5 μ L/30 sec) with IL-1ra (2 μ g/2 μ L, R&D Systems, Minneapolis, MN, USA, Catalog #280-R) or vehicle (sterile PBS with 0.1% bovine serum albumin [BSA], 2 μ L). This was repeated on day 2 and day 4 of DSS treatment, and the day after termination of DSS administration. Approximately 24h after the last infusion, mice were placed in the elevated plus maze to test for anxiety-related behavior.

Elevated plus maze (EPM)

The EPM was used for assessment of anxiety-related behavior, as we have done previously [19, 42]. Our EPM consisted of two open (6 x 30 cm, 70 lux) and two closed (6 x 30 x 15 cm, 20 lux) arms radiating from a central platform (6 x 6 cm, 55 lux) to form a plus-shaped figure. The maze was elevated 50 cm above the floor. Each mouse was placed on the central platform facing a closed arm and allowed to explore the maze for 5 min. The 5 min test period was recorded by means of a video camera and later analyzed using TopScanTM 2.0 software (Clever Sys Inc., Virginia, USA). This allowed for the calculation of the percentage time spent in the arms as well as the total distance travelled, which were deemed measures of anxiety and locomotion respectively. The maze was thoroughly cleaned before each test.

Data presentation and statistics for EPM

Each data set consists of experiments carried out in control (PBS, with 0.1% BSA) or IL-1ra -treated DSS and control mice from 2 cohorts of animals over a period of 3 months. Data are shown as individual data points as well as mean $\pm$ SEM. For statistical analysis, GraphPad Prism was used, and differences compared between control- vs DSS-treated animals by a 2-way ANOVA (factor treatment x drug), followed by Bonferroni post hoc test for % time in the open arms, closed arms and total distance travelled. A total of 37 animals were used in 2 cohorts. One mouse was eliminated due to technical difficulties and lack of video, while 3 outliers were identified using the Grubbs' test. A P value of ≤ 0.05 was considered significant.

Results

We conducted the majority of our studies in female mice. We determined that mice had colitis based on the degree of macroscopic damage, using previously published methods [19, 23, 43]. In female mice, the macroscopic damage score after DSS administration was significantly increased (4.1 ± 0.3 ; $n=10$; $t=10.6$, $df\ 18$, $P < 0.001$) compared to their respective controls (0.4 ± 0.1 , $n=10$). Male mice treated identically have slightly higher macroscopic damage scores than (4.9 ± 0.4 ; $n=8$; $t=6.9$, $df\ 10$, $P < 0.001$), compared to their respective controls (0.5 ± 0.3 , $n=4$). Macroscopic damage scores for all groups used in these studies are shown in Supplementary Table 1.

It has previously been shown that neutrophils and monocytes traffic to the brain in DSS colitis [20]. To determine a possible mechanism, we first examined the expression of $\alpha 4\beta 7$ integrin on circulating peripheral blood monocytes and neutrophils from control and DSS treated female mice, 7 days after the induction of colitis. In peripheral blood, the frequencies of monocytes and neutrophils were not significantly different between controls and animals with colitis, representing about 7% and 50% of circulating leukocytes, respectively (Figure 1, Supplementary Figure 1). Sequential gating was then used for the flow cytometric identification of $\alpha 4\beta 7$ expressing monocytes and neutrophils from peripheral blood (Figure 1). The percentage of classical monocytes was significantly increased in peripheral blood of mice with DSS colitis and the percentage of circulating inflammatory $Ly6C^{hi}$ monocytes expressing $\alpha 4\beta 7$ integrin was also significantly increased (Figure 1). In contrast, a low level of $\alpha 4\beta 7$ expression was found on neutrophils. The frequency of $\alpha 4\beta 7$ positive neutrophils increased slightly in the peripheral blood of DSS colitis mice compared with controls (Figure 1).

We next used intravital microscopy to investigate the recruitment of immune cells to the cerebral vasculature of female mice with colitis [17, 20]. An increase in the number of rolling and adherent leukocytes on cerebral endothelial cells was observed (Figure 2 and Supplementary Video 1). In male mice with colitis, a similar increase in the number of rolling and adherent leukocytes along cerebral endothelial cells was measured (Supplementary Figure 4).

We then investigated the involvement of integrins in the mechanism of recruitment of leukocytes to cerebral endothelial cells in colitis. Treatment with anti- $\alpha 4$ integrin in mice with colitis 18-22h prior to the imaging experiment significantly reduced the number of rolling and adherent leukocytes compared to colitic mice treated with the isotype control (Figure 3A, 3B), without altering the degree of colitis (Supplementary Table 1). Similarly, colitic mice treated with anti- $\alpha 4\beta 7$ displayed a significant reduction in the number of rolling leukocytes along cerebral endothelial cells (Figure 3C, 3D) and a tendency for a reduction in adherent leukocytes (Figure 3C; Colitis + IgG2a: 1.8 ± 0.8 cells/100 μ m/min; colitis + anti- $\alpha 4\beta 7$: 0.4 ± 0.3 cells/100 μ m/min; $t=2.0$, $df\ 14$, $P = 0.07$). The $\alpha 4\beta 7$ integrin binds to the adhesion molecule MAdCAM-1 expressed on endothelial cells. In mice with colitis treated with a monoclonal antibody against MAdCAM-1, the number of rolling leukocytes along cerebral endothelial cells was also significantly reduced (Figure 3E, 3F, Supplementary Video 2), and a tendency for a reduction in adherent leukocytes was also observed (Figure 3E; Colitis + IgG2a: 1.6 ± 0.4 cells/100 μ m/min; colitis + anti-MAdCAM-1: 0.7 ± 0.3 cells/100 μ m/min; $t=1.8$, $df\ 9$, $P = 0.11$).

In order to investigate the interaction of circulating neutrophils with cerebral endothelial cells in colitis, we used a PE-conjugated Ly6G antibody to visualize neutrophils during intravital microscopy. In female mice with colitis, the number of Ly6G-PE positive cells interacting with cerebral endothelial was greater than in control mice (Figure 4, Supplementary Video 3). These observations were confirmed in male mice with colitis (Supplementary Figure 4). To further examine their involvement, we used anti-Ly6G to deplete neutrophils in mice with colitis. Using flow cytometry, we confirmed that the Ly6G cells were depleted following an injection 200µg of anti-Ly6G (Supplementary Figure 3). Intravital microscopy showed that the number of both rolling and adherent leukocytes interacting with the cerebral endothelial cells of mice injected with anti-Ly6G with colitis was markedly reduced compared to colitic mice injected with isotype IgG control (Figure 5).

We then extended these findings to examine the role of monocytes, since neutrophils themselves have low levels of $\alpha 4\beta 7$ expression (Figure 1). In order to investigate the interaction of circulating monocytes with cerebral endothelial cells in colitis, we used an APC-conjugated Ly6C antibody to visualize monocytes during intravital microscopy (Figure 6). We then used anti-Ly6C to deplete monocytes in mice with colitis. Using flow cytometry, we confirmed depletion of Ly6C^{hi} classical monocytes, but not neutrophils (Supplementary Figure 3). The number of rolling and adherent Ly6C-APC positive cells, (i.e. monocytes), was reduced in mice treated with anti-Ly6C (Figure 6). We then treated mice with anti-Ly6C to deplete monocytes and examined the number of Ly6G-PE positive cells (i.e. neutrophils), interacting with cerebral endothelial cells. Remarkably, we found the number of rolling and adhering neutrophils was significantly reduced when mice were treated with anti- Ly6C compared to those treated with the isotype control (Figure 6, Supplementary Video 4).

In order to determine if leukocyte - cerebral endothelial cell interactions initiate neuroimmune activation, we measured cytokine levels in the cortex of DSS-treated mice. It has previously been shown that IL-1 β levels are increased in the cortex of DSS-treated mice [20]. We confirmed and extended these findings by demonstrating that treatment with anti- $\alpha 4\beta 7$ significantly reduced IL-1 β levels (Figure 7A). In addition, we measured C-C motif chemokine ligand 2 (CCL2), since this has previously been shown to be elevated in the brain in a mouse model of immune-mediated liver inflammation,[41] and is a monocyte chemoattractant. CCL2 was significantly elevated in the brain in colitic mice, and treatment with anti- $\alpha 4\beta 7$ significantly reduced CCL2 levels (Figure 7B). In contrast, other cytokines that were elevated in the brain in mice with colitis (GM-CSF, IL-2, IL-6, IL-12(p70) [Supplementary Table 2] and IL-10, Figure 7C) were unaffected by treatment with anti- $\alpha 4\beta 7$.

Finally, to determine the functional significance of the elevated levels of IL-1 β , we assessed anxiety-like behaviour using the elevated plus maze and blocking IL-1 β with an ICV infusion of the IL-1 receptor antagonist (IL-1ra). Administration of IL-1ra significantly increased the amount of time spent in the open arms ($F(1,29) = 5.0$, $P = 0.03$), and subsequently decreased closed arm time ($F(1,29) = 4.0$, $P = 0.05$) in animals with colitis, compared with their respective phosphate-buffered saline (PBS)-treated controls (Figure 8). Furthermore, a significant interaction effect was evident when we examined open arm (factor treatment x drug: $F(1,29) = 6.6$, $P = 0.02$) and closed arm time (factor treatment x drug: $F(1,29) = 6.0$, $P =$

0.02). Post hoc comparisons revealed that colitic mice treated with IL-1ra spent more time in the open arms (Figure 8A), and less time in the closed arms (Figure 8B), when compared with PBS-treated DSS mice. IL-ra treatment did not affect overall distance travelled in the maze (Figure 8C).

Discussion

Patients with IBD exhibit a higher prevalence of cognitive, behavioural and emotional disorders when compared to the general population [6-10]. However, the mechanisms underlying these changes in the brain remain poorly understood. Here using an acute model of colitis, we identified a novel mechanism whereby classical monocytes within the circulation adhere to the cerebral endothelium, through integrin $\alpha 4 \beta 7$ -MAdCAM-1 interactions, which directs the recruitment of neutrophils to the brain vasculature, leading to increased expression of IL-1 β that mediates anxiety-like behaviour. These exciting observations may explain, at least in part, the behavioral benefits of anti-integrin monoclonal antibody, vedolizumab in patients with IBD [38] and identify new potential targets for the treatment of the maladaptive behaviors that commonly affect IBD patients, even when their disease is in clinical remission.

In contrast to physical symptoms of IBD, the psychological manifestations of these disorders demonstrate only modest improvements over long-term follow-up [44, 45], contribute to poor outcomes [46], and are associated with increased mortality [47]. Current therapies that alleviate physical symptoms and induce disease remission have little impact on IBD-associated psychological symptoms [48-51]. In fact, these changes often go undiagnosed and are poorly managed [52-54]. Despite their high prevalence, the etiology of such symptoms in patients is poorly understood. However, to mediate changes within the CNS that alter behavior, communication pathways must exist between the inflamed gut and the brain. To date, neural, humoral (i.e. circulating gut-derived mediators) and microbial signaling pathways from the gut to the brain have been most widely studied as underlying mechanisms regulating colitis-associated behavioral changes [55-60]. Our group has previously identified a role for the cerebral recruitment of activated classical monocytes from the circulation to the brain as an important mechanism linking liver inflammation to neuroinflammation, and subsequent changes in brain function and behavior [16, 17, 41]. Specifically, we showed that the rolling and adhesion of classical monocytes to cerebral endothelium enhanced levels of proinflammatory cytokines and the chemokine CCL2 in the brain, which critically regulated the subsequent migration of monocytes into the brain to drive inflammation-associated development of maladaptive behaviors [16, 17, 41]. In our current study, we have extended our previous observations in experimental liver disease to a model of acute colitis and found important differences between these models. Previously, we showed that monocytes were exclusively recruited to the brain vasculature in mice with liver inflammation, not neutrophils, and this recruitment led to increased levels of tumor necrosis factor (TNF) and CCL2 within the CNS [41]. Our current findings suggest that acute gut and liver inflammatory processes differentially impact neuroimmune responses within the brain. Specifically, neutrophils, not monocytes, represent the predominant leukocyte recruited to the brain during acute colitis, and the resulting adhesive interactions with the cerebral endothelium drives an increased expression of CCL2 and IL-1 β in the brain.

Our findings are in agreement with a previous intravital microscopy study in mice where it was demonstrated that leukocytes are recruited to the vasculature of the prefrontal cortex during the recovery phase of DSS colitis (7 days after discontinuation of DSS)[20]. In this study, rolling leukocytes consisted of similar numbers of neutrophils and monocytes, and classical monocytes were the predominant adherent leukocyte. We studied a more acute phase of colitis and found that at this time point neutrophils were the predominant leukocyte subtype, with monocytes representing only a minor cell population. These observations imply that recruitment of different leukocyte populations to the brain vasculature during peripheral inflammatory processes involving different organ systems or timelines, may differentially impact neuroimmune responses within the brain microvasculature.

The role of neutrophils recruited to the brain in colitis was the subject of a recent study in mice in the recovery phase of acute colitis (9 days after discontinuing DSS) [58]. Here it was shown that neutrophils, but not monocytes, regulate neuronal excitability and that the reduction in seizure thresholds were mediated by TNF. However, when examining the brain parenchyma, both neutrophils and monocytes are significantly elevated, both in this late acute phase and during chronic colitis [58]. Interestingly, they found that infiltrating neutrophils were the source of TNF.

Classically, neutrophils are considered the first cells recruited to inflammatory sites and they subsequently mediate the recruitment of monocytes [61]. However, a number of studies have clearly shown that early monocyte recruitment is essential in some scenarios to promote subsequent neutrophil adhesion to endothelium and ultimate robust neutrophil recruitment into inflammatory sites [62-64]. This neutrophil adhesion-enhancing effect of monocytes at the level of the endothelium was shown to be mediated by early monocyte adhesion that induced endothelial cell activation and subsequent increased endothelial expression of neutrophil binding ligands ICAM-1 and E-selectin [65]. Moreover, this pro-neutrophil adhesive effect occurred at physiological circulating monocyte levels and did not depend on cell-cell interactions [65]. Similarly, we now show that monocyte recruitment critically regulates subsequent neutrophil recruitment to the brain in the setting of DSS colitis. However, in contrast to earlier observations, we found that monocyte-cerebral endothelial adhesive interactions regulate subsequent neutrophil recruitment via endothelial cell upregulation of MadCAM-1 expression. Moreover, this cell adhesive process and neutrophil recruitment is critically dependent on $\alpha 4\beta 7$ -MAdCAM-1 interactions. The mechanism linking monocyte recruitment to subsequent neutrophil recruitment is currently unclear but may lie in the production of a neutrophil chemoattractant that is enhanced during monocyte - endothelial adhesive interactions.

Previous work has shown that cerebral endothelial expression of adhesion molecules involved in leukocyte recruitment is upregulated in models of colitis in both rats and mice.[66] However, MAdCAM-1 is not thought to be expressed on murine cerebral endothelial cells either constitutively or in IL-10 knockout mice during inflammation [67, 68]. Though its' expression can be induced in brain endothelial cells [69] and it has been demonstrated by electron microscopy in the inflamed spinal cord of mice [70]. In human brain, MAdCAM-1 has been cloned and various alternatively splice variants have also been identified [71], but its function there remains to be determined. Further studies are required to determine

where $\alpha 4\beta 7$ expressing monocytes and MAdCAM-1 are interacting to prime the neutrophils that subsequently enter the brain in this model of colitis.

Inflammatory effector lymphocytes expressing $\alpha 4\beta 7$ are recruited to the bowel in IBD patients, and this observation precipitated the development of $\alpha 4\beta 7$ blockade using vedolizumab as an effective treatment strategy in IBD [34, 35]. However, it has become increasingly clear that $\alpha 4\beta 7$ is also expressed on monocytes, in both mice and humans, and regulates the trafficking of both inflammatory and repair monocytes to the bowel [36, 37]. In keeping with these previous observations, we have identified a significant $\alpha 4\beta 7$ -expressing monocyte population in the peripheral blood of mice with DSS colitis. Interestingly, we did not find an impact of $\alpha 4\beta 7$ blockade on DSS colitis severity, possibly because it was administered after the colitis was already established.

In mice with liver inflammation, TNF played the central role in regulating the expression of maladaptive behaviors [17] and we have also demonstrated a role for TNF in regulating neuronal excitability in rats with colitis induced by trinitrobenzene sulphonic acid [27, 28]. As noted above, in the recovery phase of acute DSS colitis, TNF mediates changes neuronal hyperexcitability [58]. However, we found that IL-1 β is elevated in the acute DSS model of colitis in mice, as it is in the hippocampus of mice with chronic DSS colitis [72]. The magnitude of the changes in IL-1 β levels we observed are similar to that observed in the hippocampus in chronic colitis [72] and in febrile seizures [73]. Increased levels of the proinflammatory cytokine IL-1 β in the brain induces a number of behavioral changes, including anxiety-like behaviors [74, 75], and inhibition of IL-1 β -mediated effects in the brain attenuates these behavioral changes [76, 77]. The source of increased brain IL-1 β levels in DSS-treated mice remains unknown. In chronic mouse stress anxiety models, monocytes are recruited to cerebral endothelial cells by microglia produced CCL2, and these recruited monocytes produce IL-1 β which subsequently activates cerebral endothelial cells via IL-1R1 to generate anxiety-like behavior [78]. However, there are other potential sources including microglia, astrocytes and neurons [79-81].

In accord with previous observations from our laboratories [19] and elsewhere [20-25], we show that mice with DSS colitis demonstrate anxiety-like behaviors. In addition, similar to observations by Gadotti *et al.* [20], we show that DSS colitis also induces IL-1 β expression in the brain. Here we provide novel information that this colitis-associated increase in IL-1 β expression is driven by $\alpha 4\beta 7$ -mediated leukocyte adhesive interactions with cerebral endothelium. Moreover, we now link these observations by demonstrating that blocking IL-1 β -mediated effects in the brain using IL-1ra attenuates the development of anxiety-like behaviors in colitic mice. Given the frequent association of maladaptive behavioral changes, including anxiety, in IBD patients [6, 7, 9], our new observations suggest that this leukocyte-cerebral endothelium – IL-1 β axis may represent an opportunity to develop novel targets for treating IBD-associated behavioral alterations.

Conclusions

In conclusion, by showing that $\alpha 4\beta 7$ integrin expressing monocytes direct the recruitment of neutrophils to the brain in acute colitis, we have identified a new mechanism that helps explain how peripheral inflammation alters activity in the CNS, which ultimately leads to changes in behavior. In this study, we examined anxiety-like behavior and demonstrated that this was mediated by the proinflammatory cytokine IL-1 β . In future studies, other behaviors, such as depression and fatigue should be assessed to build a more comprehensive understanding of the role of this novel mechanism in mediating the behavioral comorbidities of colitis.

List Of Abbreviations

APC, allophyocyanin; BSA, bovine serum albumin; CCL2, C-C motif chemokine ligand 2; CNS, central nervous system; DSS, dextran sodium sulfate; GI, gastrointestinal; IBD, inflammatory bowel disease; ICV, intracerebroventricular; IL, interleukin 1; IL-1ra, interleukin 1 receptor antagonist; IP, intraperitoneal; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PBS, phosphate buffered saline; PE, phycoerythrin; TNF, tumor necrosis factor.

Declarations

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Contributions

NLC, KDN, WA, SAH, QJP, MGS and KAS designed the studies; NLC, KDN, WA, BHL, and LG conducted the experiments and performed data analyses; NLC, MGS and KAS drafted the manuscript. All authors had access to the study data and critically reviewed and approved the final manuscript for submission. SAH, QJP, MGS and KAS obtained funding for the study and provided study supervision.

Ethics declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Health Sciences Animal Care Committee of the University of Calgary and were carried out in accordance with the guidelines of the Canadian Council on Animal Care (Protocol #'s AC17-0093, AC15-0129).

Consent for publication

Not applicable

Competing interests

The authors declare no conflicts of interest exist.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Colitis increases the percentage of classical monocytes and induces the upregulation of $\alpha 4\beta 7$ integrin on circulating classical monocytes and neutrophils. The expression of $\alpha 4\beta 7$ integrin on the surface of circulating peripheral blood monocytes and neutrophils from control and colitic mice was assessed by multicolor flow cytometry. A, B. The frequencies of monocytes and neutrophils were not significantly altered between mice with colitis and controls (monocytes, $t=1.8$, df 8, $P=0.11$; neutrophils, $t=0.01$; df 8, $P=0.99$, $n=5$ mice/group). C. Higher percentages of total classical monocytes were detected in the peripheral blood of colitic mice compared to control group ($t=4.9$, df 8, $***P < 0.001$; $n=5$ mice/group). D, E. There was a significant upregulation of $\alpha 4\beta 7$ expression on the surface of classical monocytes in colitic mice. D. Percentage of $\alpha 4\beta 7^+$ classical monocytes ($t=3.4$, df 8, $**P=0.01$; $n=5$ mice/group). E. Cell surface $\alpha 4\beta 7$ expression on classical monocytes expressed as mean fluorescent intensity $t=5.6$, df 8, $***P < 0.001$; $n=5$ mice/group). F. Circulating blood neutrophils of control mice expressed very low levels of $\alpha 4\beta 7$ which increased significantly in colitic mice ($t=32.7$, df 8, $***P < 0.001$; $n=5$ mice/group).

Figure 2

Colitis induces the rolling and adhering of leukocytes in cerebral endothelial cells. Intravital microscopy was performed using a spinning disc confocal microscope with a 20X/0.95 NA water objective. Videos were captured and analyzed to identify rolling and adhering leukocytes in control and colitic mice. A. Colitic mice showed a significant increase in rolling ($t=2.8$, $df\ 11$, $*P=0.02$; $n=6-7$ mice/group) and adhering ($t=3.1$, $df\ 11$, $*P=0.01$; $n=6-7$ mice/group) leukocytes on cerebral endothelial cells. B. Representative images of intravital imaging. CD31 was to label cerebral endothelial cells (blue), Rho6G was used to label leukocytes (red). Scale bar: 25 μm .

Figure 3

Anti-integrins block the rolling and adhering of leukocytes on cerebral endothelial cells during colitis. Intravital microscopy was used to identify rolling and adhering leukocytes in colitic mice treated with an anti-integrin or an isotype control 18-22h prior to imaging. A. Anti- $\alpha 4$ integrin significantly reduced rolling ($t=2.9$, $df\ 14$, $*P=0.01$, $n=8$ mice/group) and adhering ($t=4.3$, $df\ 14$, $***P<0.001$, $n=8$ mice/group) leukocytes in colitic mice compared to isotype-treated colitic controls. B. Representative images from intravital microscopy. CD31 was used to label cerebral endothelial cells (blue), Rho6G was used to label leukocytes (red). C. Anti- $\alpha 4\beta 7$ significantly reduced rolling leukocytes in colitic mice ($t=2.9$, $df\ 12$, $*P=0.01$, $n=6-8$ mice/group) but not adhering ($t=2.0$, $df\ 12$, $P = 0.07$, $n=6-8$ mice/group) compared to isotype-treated colitic controls. D. Representative images from intravital microscopy. CD31 was used to label cerebral endothelial cells (blue), Rho6G was used to label leukocytes (red). E. Anti-MAdCAM-1 significantly reduced rolling leukocytes in colitic mice ($t=2.4$ $df\ 9$, $*P = 0.04$, $n=5-6$ mice/group) but not adhering ($t=1.8$, $df\ 9$, $P=0.11$, $n=5-6$ mice/group) compared to isotype-treated colitic controls. F. Representative images from intravital microscopy. CD31 was used to label cerebral endothelial cells (blue), Rho6G was used to label leukocytes (red). Scale bar for B, D, F: 25 μm .

Figure 4

Colitis induces the rolling and adhering of neutrophils on cerebral endothelial cells during colitis. Intravital microscopy was used to identify rolling and adhering neutrophils (Ly-6G positive cells) in control and colitic mice. A. Colitis significantly increased the rolling ($t=2.8$, df 10, $*P=0.01$; $n=6$ mice/group) and adhering ($t=2.7$, df 10, $*P<0.02$; $n=6$ mice/group) neutrophils in colitic mice compared to controls. B. Representative images from intravital microscopy. CD31 was used to label cerebral endothelial cells (blue), Ly6G was used to label neutrophils (red). Scale bar: 25 μ m.

Figure 5

Blocking neutrophils reduces the rolling and adhering of leukocytes on cerebral endothelial cells during colitis. Intravital microscopy was used to visualize the rolling and adhering of leukocytes in colitic mice treated with anti-Ly6G to block neutrophils compared to isotype controls. A. Anti-Ly6G significantly reduced both rolling ($t=4.0$, df 11, $**P<0.01$; $n=5-8$ mice/group) and adhering ($t=2.3$, df 11, $*P=0.04$; $n=5-8$ mice/group) leukocytes in colitic mice compared to isotype-treated colitic controls. B. Representative images from intravital microscopy. CD31 was used to label cerebral endothelial cells (blue), Rho6G was used to label leukocytes (red). Scale bar: 25 μ m.

Figure 6

Anti-Ly6C depletes monocytes and depleting monocytes reduces the rolling and adhering of neutrophils on cerebral endothelial cells during colitis. Intravital microscopy was used to visualize the rolling and adhering of neutrophils and monocytes in colitic mice treated with anti-Ly6C to block monocytes or treated with an isotype control. A. Colitic mice treated with anti-Ly6C significantly reduced rolling ($t=8.5$, df 8, $***P<0.001$; $n=4-6$ mice/group) but not adhering ($t=0.1$, df 8, $P=0.89$; $n=4-6$ mice/group) monocytes

(Ly6C positive cells) compared to colitic mice treated with an isotype control. B. Anti-Ly6C treatment that reduces monocytes, significantly reduced rolling ($t=3.3$, $df\ 9$, $**P=0.009$; $n=5-6$ mice/group) and adhering ($t=2.7$, $df\ 9$, $*P=0.02$, $n=5-6$ mice/group) neutrophils in colitic mice compared to colitic mice treated with an isotype control. C. Representative images from intravital microscopy. Ly6C was used to label monocytes (blue), Ly6G was used to label neutrophils (red). Scale bar: 25 μm .

Figure 7

In vivo neutralization of $\alpha 4\beta 7$ integrin modulates colitis-induced increases in cortical cytokine levels. To address the possible role of the $\alpha 4\beta 7$ integrin in initiating neuroimmune activation, cortical cytokine levels of colitic mice were assessed after anti- $\alpha 4\beta 7$ treatment. Increased cortical cytokines levels of (A) IL-1 β and (B) CCL2 induced by colitis were significantly reduced by anti- $\alpha 4\beta 7$ integrin antibody treatment ($F(2, 15)=4.79$, $*P=0.02$ and $F(2, 15)= 5.08$, $*P=0.02$, respectively; colitis + anti- $\alpha 4\beta 7$ vs. all other groups, one-way ANOVA; $n=6$ mice per group). C. $\alpha 4\beta 7$ integrin neutralization did not alter colitis-induced changes in cortical IL-10 levels ($F(2, 13) = 6.45$, $*P=0.01$; colitis + anti- $\alpha 4\beta 7$ and colitis + isotype vs. control group, one-way ANOVA; $n=6$ mice per group).

Figure 8

Interleukin-1 receptor antagonist (IL-1ra) reduces the anxiety-like phenotype in colitic mice but does not affect locomotion. To investigate the effect of colitis and IL-1ra on behavior, control and colitic mice were implanted with intracerebroventricular cannulas above the right lateral ventricle and infused with either PBS or IL-1ra. At peak colitis, mice were assessed for anxiety-like behavior using the elevated plus maze. A. In colitic mice, IL-1ra significantly increased the percentage time spent in the open arms of the maze

($F(1, 29) = 5.0$, $*P=0.01$; $n=7-10$ mice/group, two-way ANOVA). B. In colitic mice, IL-1ra significantly decreased the percentage time spent in the closed arms of the maze ($F(1, 29) = 4.0$, $*P=0.021$; $n=7-10$ mice/group, two-way ANOVA) compared to colitic mice infused with PBS, indicating a decrease in anxiety-like behavior. C. Total distance traveled in the elevated plus maze remained unchanged across each treatment condition ($F(1, 29) = 0.12$, $P=0.73$; $n=7-10$ mice/group).

Supplementary Files

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